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Enzyme Inhibitors of Microbial Origin [and Discussion]

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Enzyme inhibitors of microbial origin

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The screening for enzyme inhibitors of microbial origin in the past decades has been a prosperous area to find new metabolites that are of potential importance as therapeutic or antibiotic agents. This review attempts a survey of recent achievements in this type of screening. Special emphasis is given to enzyme inhibitors and screening systems in fields where industry has a main interest in development. This includes some notes on the improved methodology for the detection of reversible and irreversible inhibitors of β-lactamases and the presentation of a unique inhibitor of α-amylase from porcine pancreas isolated from a strain of *Streptomyces tendae*. This inhibitor (HOE 467) may be of potential use in the treatment of diabetic conditions, obesity and adipositas. The results show that the screening for enzyme inhibitors from microorganisms still provides one of the central challenges for future research.

Introduction

The study of the effects of inhibitors on the activity of isolated enzymes and on metabolic sequences in general has been of the greatest importance for the elucidation of the biochemical and regulatory aspects of life. Besides their use as scientific tools, enzyme inhibitors have attained much attention as compounds that are or may be of potential use as chemotherapeutic agents or pharmacologically active drugs. A sign of the advances in this highly dynamic field is the number of review articles published on the subject, covering enzyme inhibitors from microbial sources as well as inhibitors that have been designed by medicinal chemists (Umezawa 1977; Brannon & Fuller 1974; Aszalos & Berdy 1978; Jung 1978; Fisher & Knowles 1978).

Now 14 years have passed by since Umezawa and his group, using culture broths of microorganisms, started a systematic screening for enzyme inhibitors. Umezawa's courageous approach in 1965 was based on the knowledge that microorganisms produce a nearly unconceivable number of different antibiotics and other secondary metabolites, some of which had already been shown at this time to inhibit single enzyme reactions *in vitro*. Even though, in 1972, he commented that he had originally had 'the intention of finding useful tools for biochemical functions and diseases' (Umezawa 1972), it can be assumed that, in fact, he had more practically orientated goals in mind when launching this programme.

The current screening of enzyme inhibitors in 1979 is characterized by two main research areas:

1. enzyme inhibitors of pharmacological interest;
2. inhibitors of β-lactamases.

These will be covered separately in the following two sections.

Enzyme inhibitors with pharmacological effects

General aspects

Enzyme inhibitors of pharmacological interest means, of course, enzyme inhibitors useful as drugs. The chemical approach along the line of the classical antimetabolite concept, which has been followed ever since 1940, introduced small structural changes into metabolites. However, the number of clinically successful compounds that have emerged from this chemical approach has been disappointingly small, mainly as a consequence of the competitive nature of inhibition displayed by this type of structural analogue.

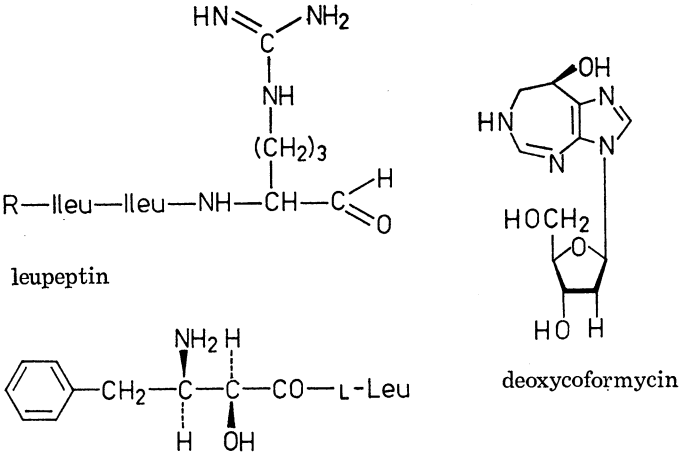

Figure 1. Structural formulae of leupeptin, bestatin and deoxycoformycin.

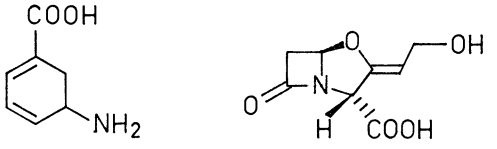

Figure 2. Structural formulae of gabaculine and clavulanic acid.

In the course of the systematic screening for enzyme inhibitors in microbial cultures, more than 50 novel compounds with pharmacological activities in various areas have been reported up to now in the literature. One common feature of these inhibitors is that in many cases they hardly bear any structural resemblance with the normal substrate of the enzyme, which may reflect the fact that microorganisms are ignorant when it comes to patent literature.

It is beyond the scope of this report to discuss the structural and pharmacological details of all these compounds. It is, however, worth noting that many inhibitors display entirely new mechanistic features. This is illustrated for a few selected inhibitors in figures 1 and 2. Leupeptin, bestatin and deoxycoformycin can be regarded as transition state analogues (Jung 1978) that are highly effective inhibitors in the 10^{-7} to 10^{-9} M range, never reached by competitive inhi-

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bitors. Finally, gabaculine and clavulanic acid belong to the potent group of enzyme-activated irreversible inhibitors, also known as k_{cat} inhibitors or suicide enzyme inactivators (Jung 1978; Fisher *et al.* 1978).

The study of these inhibitors in terms of mechanism has contributed much to the understanding of enzyme reactions as well as to the chemical design of new compounds, and it seems not unlikely that the mechanistic types of inhibitors depicted here will govern the future of enzyme inhibitors as drugs.

TABLE 1. PHARMACOLOGICALLY ACTIVE ENZYME INHIBITORS FROM MICROORGANISMS THAT ARE IN CLINICAL USE OR IN CLINICAL TRIALS

compound	inhibits	clinical entity
fusaric acid (analogues)	dopamine-β-hydroxylase	hypertension
ML-236 B (citrinin)	HMG-CoA reductase	hyperlipidaemia
BAY g 5421	α-amylase	obesity
bestatin	aminopeptidase B	tumours
pepstatin	pepsin cathepsin D	stomach ulcers
leupeptin	plasmin trypsin papain cathepsin B	anticoagulant

Up to now, the only clinically used enzyme inhibitor of microbial origin is fusaric acid, of which some chemical analogues are also used. A list of other likely candidates is given in table 1. The list may not be complete but certainly reflects the current interest of the pharmaceutical industry in certain types of inhibitors, i.e. in fields where there is a firm correlation between enzyme inhibition and pharmacological effect.

It should be mentioned at this point that many research people engaged in the screening of pharmacologically active enzyme inhibitors from microbial sources are performing a delicate job. The correlation between enzyme inhibition and pharmacological effect is in many cases far from being established. The perspectives of a screening for inhibitors useful as drugs will therefore depend very much on the verification of the equation

$$\text{pharmacological effect} = f(\text{enzyme inhibition}).$$

α-Amylase inhibitors

On the basis of such a clear cut correlation between enzyme inhibition and pharmacological effect, we started a screening for inhibitors of α-amylases. In the course of this screening we succeeded in isolating an inhibitor that is a promising candidate to become a therapeutic agent as an accompanying drug in diabetes and in the treatment of prediabetes and obesity.

The concept of reducing or even abolishing starch-induced hyperglycaemia and hyperinsulinaemia by means of a selective inhibition of α-amylases has been considered for a long time and active principles have been described ever since 1933 (Chrzaszcz & Janicki 1933). Up until 1970, the most active inhibitors were isolated from plant material (Kneen & Sandstedt 1946; Miller & Kneen 1947; Hemberg & Larsson 1961; Gries *et al.* 1972).

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A list of amylase inhibitors isolated from microbial sources is given in table 2. All inhibitors are reported to act on the pharmacologically important α-amylases from pancreas and saliva. Chemically they belong to either oligosaccharides or oligopeptides. The amylase inhibitor from *S. calidus*, however, seems to have a more complex composition. Positive clinical trials have been reported for BAY g 5421 (Schmidt *et al.* 1977; Puls *et al.* 1977), which shows an interesting shift in specificity from α-amylase to saccharase inhibitory activity with decreasing chain lengths of the oligosaccharide.

TABLE 2. α-AMYLASE INHIBITORS FROM MICROORGANISMS

amylase inhibitor	first publication	chemical nature	company or institute
BAY g 5421	1971	oligosaccharide	Bayer AG
from *S. flavochr.*	1971	oligopeptide	Hayashibaba Biochem. Labs
X 2	1973	oligopeptide	Amano Pharma KK
amylostatin A	1974	oligosaccharide	Ajinomoto KK
S-AI	1975	oligosaccharide	Osaka University
from *S. calidus*	1976	complex	Rhone-Poulence Ind.
HOE 467	1977	oligopeptide	Hoechst AG

TABLE 3. CHARACTERISTIC FEATURES OF THE α-AMYLASE INHIBITOR HOE 467

(1) produced by *S. tendae*, strain 4158
(2) polypeptide
(3) r.m.m. *ca.* 7500
(4) lacks Met
(5) λ_{max} 276 nm
(6) isoelectric point pH 4.4
(7) specific for α-amylases from pancreas and saliva
(8) specific activity 1.7×10^7 A.I.U./g (pH 6.9, 37 °C)

Another unique α-amylase inhibitor, HOE 467, has been isolated, in our laboratories, as a colourless substance from *S. tendae*. Some characteristic features of this inhibitor are presented in table 3. HOE 467 is a polypeptide that can clearly be distinguished from other peptide-type amylase inhibitors by its relative molecular mass, amino acid composition and isoelectric point. Pure preparations of the inhibitor exhibit a specific activity of 1.7×10^7 A.I.U./g against the enzyme from porcine pancreas.

In addition to its high specific activity, this inhibitor is further characterized by its pronounced selectivity for α-amylases produced by pancreas or salivary glands. In the range of concentrations where mammalian α-amylases are completely inhibited, the respective enzymes from bacterial or fungal sources are not affected to any measurable extent. An effect on β-amylases has not been observed either.

Early experiments with HOE 467 revealed that its inhibitory effect increases upon preincubation with α-amylase, which is indicative of an irreversible type of interaction. As shown in figure 3, irreversible inhibition takes place in less than 15 s in the absence of substrate. In accordance with the irreversible type of inhibition, a linear relation is observed when the residual activity of porcine α-amylase after preincubation with HOE 467 is plotted against the concentration of inhibitor (figure 4).

The tight binding of HOE 467 to the set of at least three isoenzymes of α-amylase from porcine pancreas is illustrated in figure 5. In this experiment, various amounts of purified enzyme

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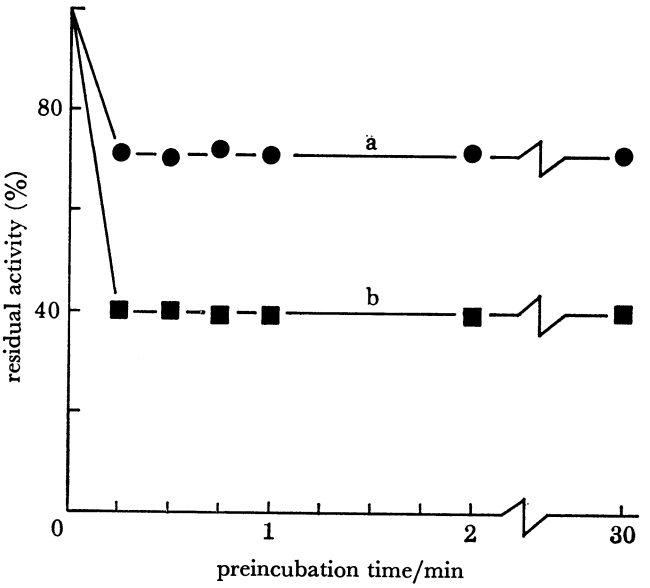

FIGURE 3. Time course of the inactivation of α-amylase from hog pancreas by HOE 467. The enzyme was pre-incubated with (*a*) 0.12, and (*b*) 0.25 μg of HOE 467. Samples were then withdrawn at the indicated times and assayed for residual activity.

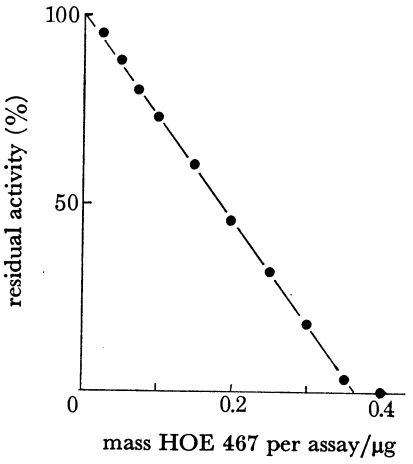

FIGURE 4. Linear relation between the residual activity of α-amylase from porcine pancreas and the amount of HOE 467 after preincubation of the enzyme with the inhibitor for 10 min.

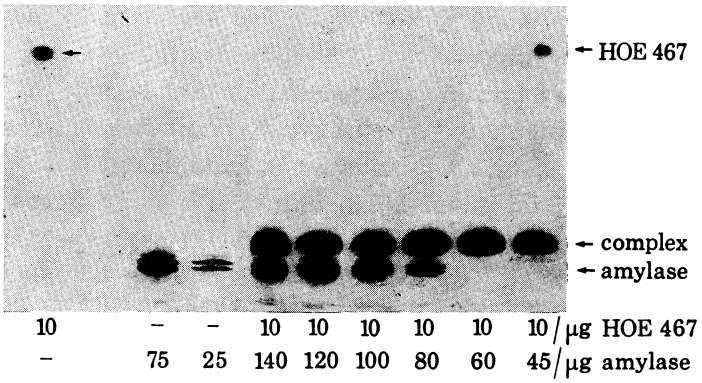

FIGURE 5. Electrophoretic mobility of HOE 467, α-amylase (porcine pancreas) and HOE 467–amylase inhibitor complexes. The indicated amounts of HOE 467 and α-amylase were preincubated for 10 min. Aliquots of this mix were subsequently subjected to gel electrophoresis. HOE 467 and α-amylase were included separately for comparison.

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were preincubated with a fixed amount of HOE 467 and subsequently subjected to gel electrophoresis. It is evident that defined isoenzyme–inhibitor complexes are formed that differ in their electrophoretic mobility from the native isoenzymes. This type of experiment further allows the calculation of the specific activity of HOE 467 on the basis of complex formation and the values thus obtained compare very well with the data from kinetic analysis.

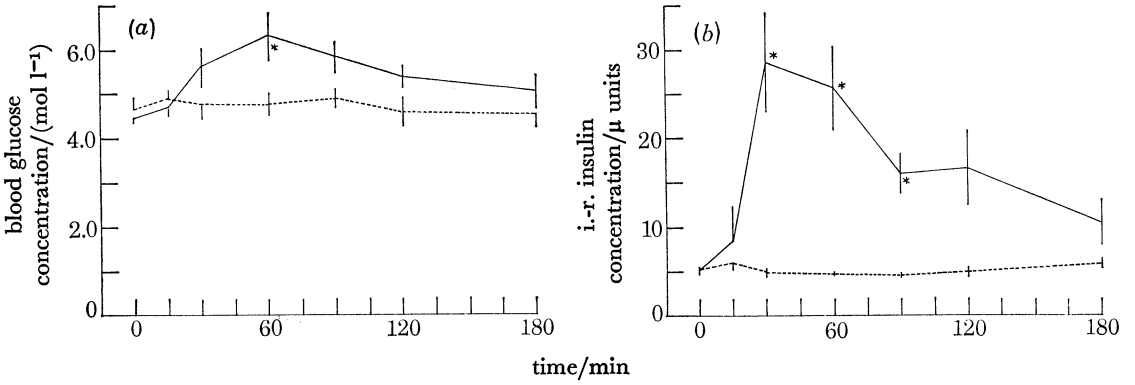

FIGURE 6. Concentrations ($\bar{X} \pm$ s.e.; $N = 4$; $*p = 0.005$): (*a*) blood glucose and (*b*) immune-reactive (i.r.) insulin in the starch-loaded dog (2 g/kg p.o.) after oral application of HOE 467 (2.5 mg/kg; dotted lines). Solid line: control.

As pointed out earlier, an α-amylase inhibitor is expected to reduce alimentary hyperglycaemia and hyperinsulinaemia. Therefore, the effect of HOE 467 on blood glucose levels and immune-reactive serum insulin in the starch-loaded dog was investigated (figure 6). Due to its reluctant response to proteolytic enzymes of the digestive tract, HOE 467 can be applied orally. In the control experiment the ingestion of starch results in a significant increase of blood glucose levels over a period of 3 h, despite a nearly sixfold increase in insulin. In the presence of the indicated amount of HOE 467, however, the starch load leaves the glucose level completely unchanged and, consequently, does not provoke a secretion of insulin. This effect is dose-dependent and not confined to this experimental animal.

Need for chromogenic substrates

The importance of a positive correlation between enzyme inhibition and pharmacological effect having been stressed, mention should be made of another screening problem. In cases where this correlation can be regarded to be a good working hypothesis, an efficient screening for enzyme inhibitors from microbial sources is often hampered by the lack of a suitable, and this means a chromogenic, substrate that can be used in the presence of crude culture broths. A good example of this is found in the screening for inhibitors of the angiotensin-converting enzyme (ACE). Such inhibitors are very effective in certain cases of hypertension as exemplified by the drug Captopril (Ondetti & Cushman 1978). Even though it is not known if ACE inhibitors have ever been looked for in microbial cultures, it is easily conceivable that the laborious analytical procedures (Spadaro *et al.* 1978) have prevented some people from doing so. Wilson and his group at the University of Colorado, however, have now shown that the activity of angiotensin-converting enzyme can be easily assayed by means of a chromogenic substrate (Persson *et al.* 1978), as shown in figure 7. The enzyme-catalysed reaction yields first the *S*-benzfurazan derivative of cysteinylglycine. A non-enzymatic internal *S* to *N* shift follows immediately to yield the *N*-benzfurazan derivative, with an attendant shift in the absorption

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maximum from 423 to 461 nm. This example of an ingeniously devised substrate makes it clear that in future we will need more mission-orientated screening groups with collaboration between people from chemistry, biochemistry, pharmacology and microbiology.

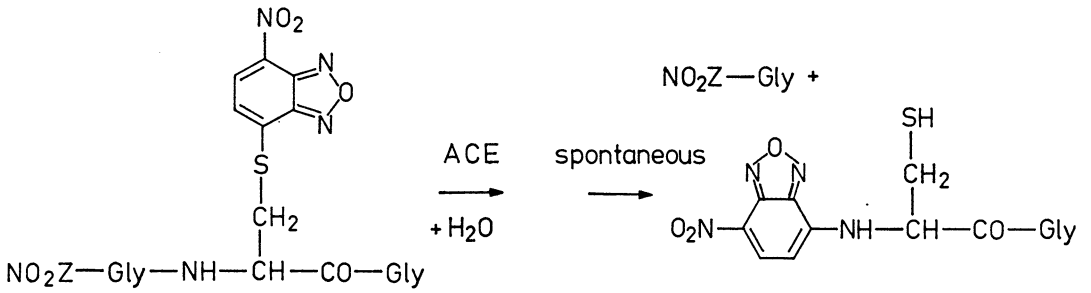

FIGURE 7. Hydrolysis of the chromogenic substrate *p*-nitrobenzyloxycarbonylglycyl-(*S*-4-nitrobenzo-2-oxa-1,3-diazole)-L-cysteinylglycine by angiotensin-converting enzyme (ACE). Addition of the thiol reagent 4,4′-dithiodipyridine improves the sensitivity of the assay (Persson *et al.* 1978).

TABLE 4. RECENTLY PUBLISHED BIOASSAYS FOR THE SPECIFIC DETECTION OF β-LACTAM COMPOUNDS

(1) β-lactam-supersensitive indicator strains
(2) use of such strains +/− β-lactamases (four-plate system)
(3) synergy between β-lactamase inhibitors and β-lactamase-sensitive antibiotic in microorganisms producing the enzyme

β-LACTAMASE INHIBITORS

The introduction of a new screening system has always proved to be a prerequisite to detection of novel types of compounds. An exciting example is the field of β-lactam antibiotics, where microbiologists are just discovering some of the most potent compounds ever produced in nature. In recent years new bioassay screening methods for the specific detection of even small amounts of β-lactams have been devised (Aoki *et al.* 1977; Kitano *et al.* 1977) (table 4). Selected results of this screening are shown in table 5, from which it is quite obvious that entirely novel β-lactam compounds could be isolated that would revolutionize the older views on structure–activity relations in the β-lactam series (Cama & Christensen 1978).

In addition to the above mentioned bioassays, β-lactam antibiotics can also be screened by simple enzymatic assays *in vitro*. According to the classification published by Richmond & Sykes (1973), β-lactamases can be differentiated from each other on the basis of whether they serve as substrates or, alternatively, act as inhibitors of the enzyme. As recently pointed out by Fisher & Knowles (1978), it is interesting enough that β-lactam antibiotics are able to inhibit both bacterial cell wall synthesis and β-lactamases.

Two types of inhibitors can be distinguished on the basis of enzyme kinetics, which is by no means of purely academic interest. The different kinetic behaviour of reversible and irreversible inhibitors is shown schematically in figure 8. If a suitable substrate, e.g. the chromogenic substrate nitrocefin (Glaxo compound 87/312) (O'Callaghan *et al.* 1972) is used, a reversible inhibitor will reduce the rate of substrate breakdown immediately. This contrasts with a progressive inhibition displayed by irreversible inhibitors, for which the shorter term 'inactivators' is proposed. Preincubation of the enzyme with an inactivator such as clavulanic acid results in the recording of the residual enzymatic activity with a linear rate and this makes the assay of β-lactamase inactivators much more sensitive. Uri and coworkers described this inactivation

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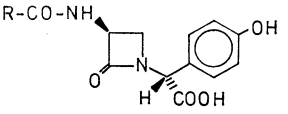
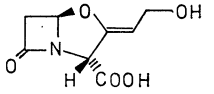
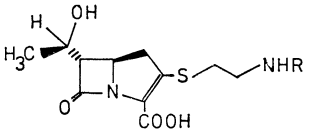
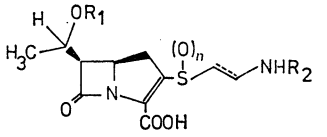
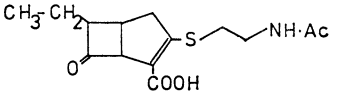
TABLE 5. STRUCTURAL FORMULAE OF NOVEL β-LACTAMS ISOLATED FROM MICROORGANISMS

basic structure	date published	compounds
	1976	nocardicins
	1976	clavulanic acid
	1976	thienamycins
	1977	olivanic acids
	1978	PS-5

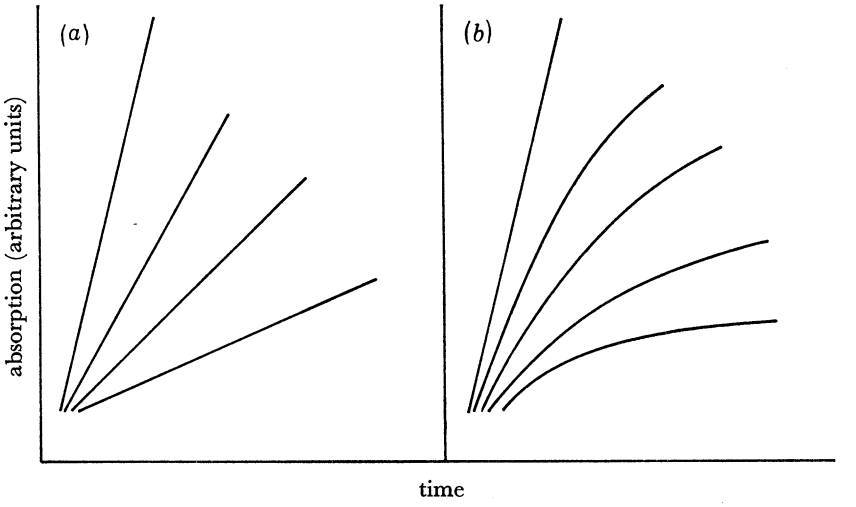


FIGURE 8. Schematic representation of different kinetic behaviour of reversible (*a*) and irreversible (*b*) inhibitors on the reaction rate of β-lactamases. Inhibitor concentration increases from upper to lower curves.

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assay with nitrocefin (Uri *et al.* 1978), and the olivanic acid-type compounds MC 696-SY2-A (identical with MM 4550) and PS-5 have been reported to be discovered and purified with the help of inactivation assays (Umezawa *et al.* 1973; Maeda *et al.* 1977; Okamura *et al.* 1978).

A screening for reversible inhibitors of β-lactamases, however, does not seem to have been reported. The kinetic assay is easy to perform and highly specific for β-lactams. The type of compounds that one may expect to pick up will have a high affinity for the binding site of the

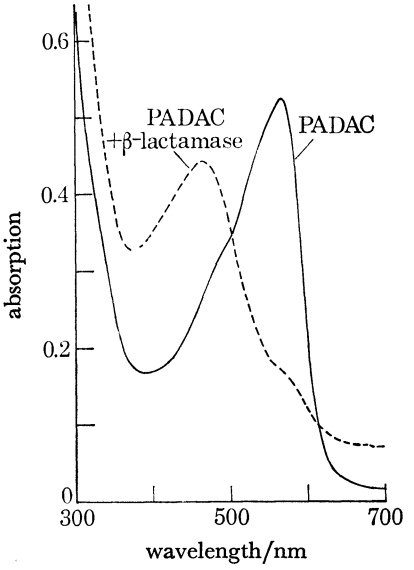

FIGURE 9. Absorption spectrum of the novel β-lactamase substrate PADAC before and after treatment with β-lactamase.

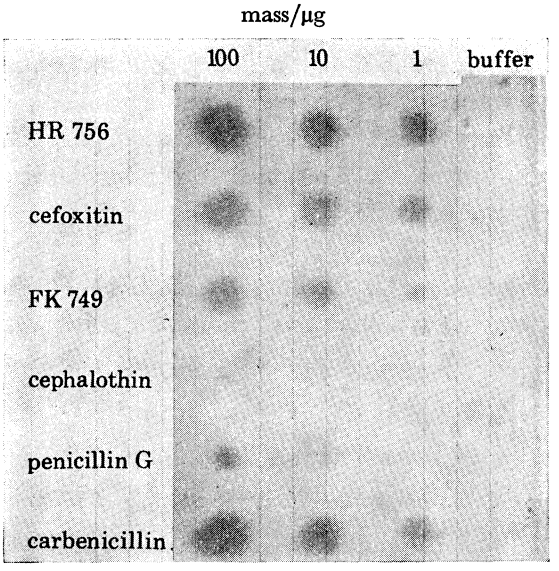

FIGURE 10. PADAC plate assay for essentially non-inactivating β-lactam compounds. Paper disks (8 mm in diameter) soaked with the indicated amount of β-lactam antibiotics were applied on top of a β-lactamase layer. After 30 min the plate was flooded with an aqueous solution of PADAC. Due to inhibition of the β-lactamase, the diffusion areas of β-lactams that are not hydrolysed during preincubation appear as violet spots on a yellow background.

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β-lactamase, which in many cases goes along with a high affinity to the target in cell wall synthesis. Such compounds will also display a fairly good resistance to hydrolysis by the enzyme used, which adds an additional positive feature to the compound. The sensitivity of this assay increases with both parameters.

For this type of screening we synthesized a series of new chromogenic β-lactamase substrates, one of which is a compound that we call 'PADAC'. This substrate is without any doubt the most highly coloured cephalosporin known. Aqueous solutions are intensely violet and turn to yellow when β-lactamases from various sources are added to the assay mixture. The corresponding shift in the absorption spectrum is shown in figure 9. This substrate is completely stable to a variety of nucleophiles such as cysteine, mercaptoethanol, dimercaptopropanolol and serum, which makes it an extremely useful substrate for screening purposes.

In an automated enzyme device both reversible and irreversible β-lactamase inhibitors can easily be detected in microbial cultures. In addition to this kinetic assay we have also developed a simple plate assay, shown in figure 10. In this experiment, essentially non-inactivating β-lactam model compounds like HR 756, cefoxitin and carbenicillin, which display a high affinity to β-lactamases but are not hydrolysed within the time needed for diffusion (30 min), can easily be detected with PADAC, in less than 1 μg amounts. This contrasts with cephalothin and penicillin G, which are hydrolysed during the preincubation. A similar procedure can be used for the localization of β-lactamase inactivators.

It can be expected that the kinetic approach to the screening of β-lactams in combination with suitable accompanying assays will result in the discovery of still more novel types of β-lactams.

Conclusion

In the course of new screening approaches, microorganisms have again given us an unexpected variety of answers to the questions that we have asked them. It seems, however, that we did not always ask the right questions in the proper way. Still, the types of compounds that have been found have led us to new heights in the understanding of life and to some promising new drugs. What we need is refinement of our questions in terms of pharmacological or chemotherapeutic relevance as well as improvement of our assay procedures; this certainly provides one of the central challenges for future research.

The author thanks Dr Neubauer, Dr Oeding, Dr Pfaff, Dr Regitz, Dr Voelskow and Dr Vertesy (Hoechst AG) for making available their results on HOE 467. Thanks are also due to Dr Blumbach, Dr Huber and Dr Schickfluss (Hoechst AG), for active participation in the preparation of PADAC.

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Discussion

B. S. Hartley, F.R.S. (*Department of Biochemistry, Imperial College, London SW7, U.K.*). Could the speaker clarify whether the screening for these inhibitors is carried out on the culture supernatants or on cell lysates?

P. Schindler. One can do either; but it is more convenient to screen the culture supernatants.

S. Brenner, F.R.S. (*M.R.C. Laboratory of Molecular Biology, Hills Road, Cambridge, U.K.*). How many of these inhibitors bind covalently?

P. Schindler. Several: for instance, clavulanic acid. Some of the inhibitors are activated by the enzyme itself to form the nucleophilic warhead.

S. Brenner. What about the oligopeptide inhibitors?

P. Schindler. They probably act as transition state analogues.

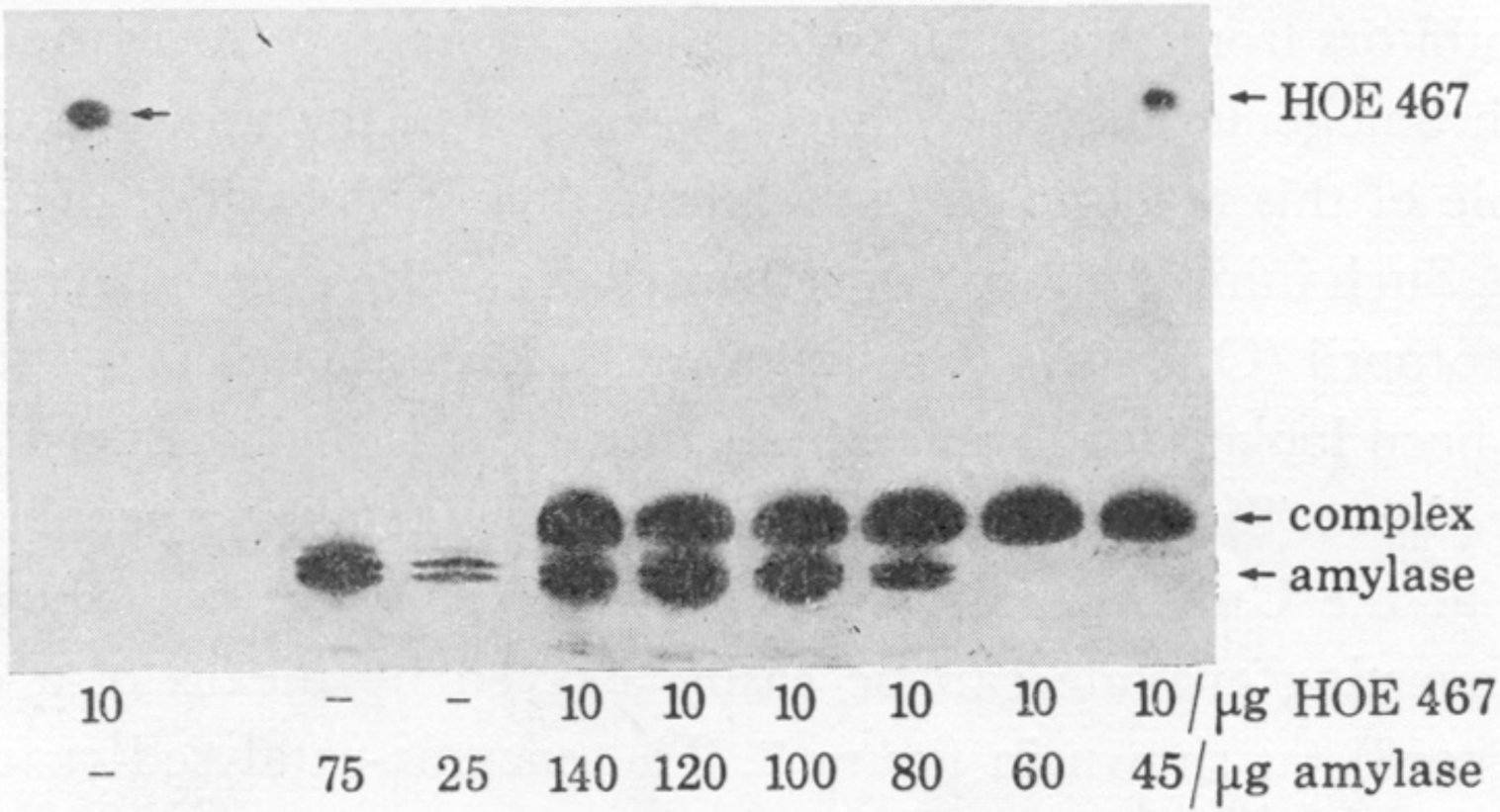

FIGURE 5. Electrophoretic mobility of HOE 467, α-amylase (porcine pancreas) and HOE 467–amylase inhibitor complexes. The indicated amounts of HOE 467 and α-amylase were preincubated for 10 min. Aliquots of this mix were subsequently subjected to gel electrophoresis. HOE 467 and α-amylase were included separately for comparison.

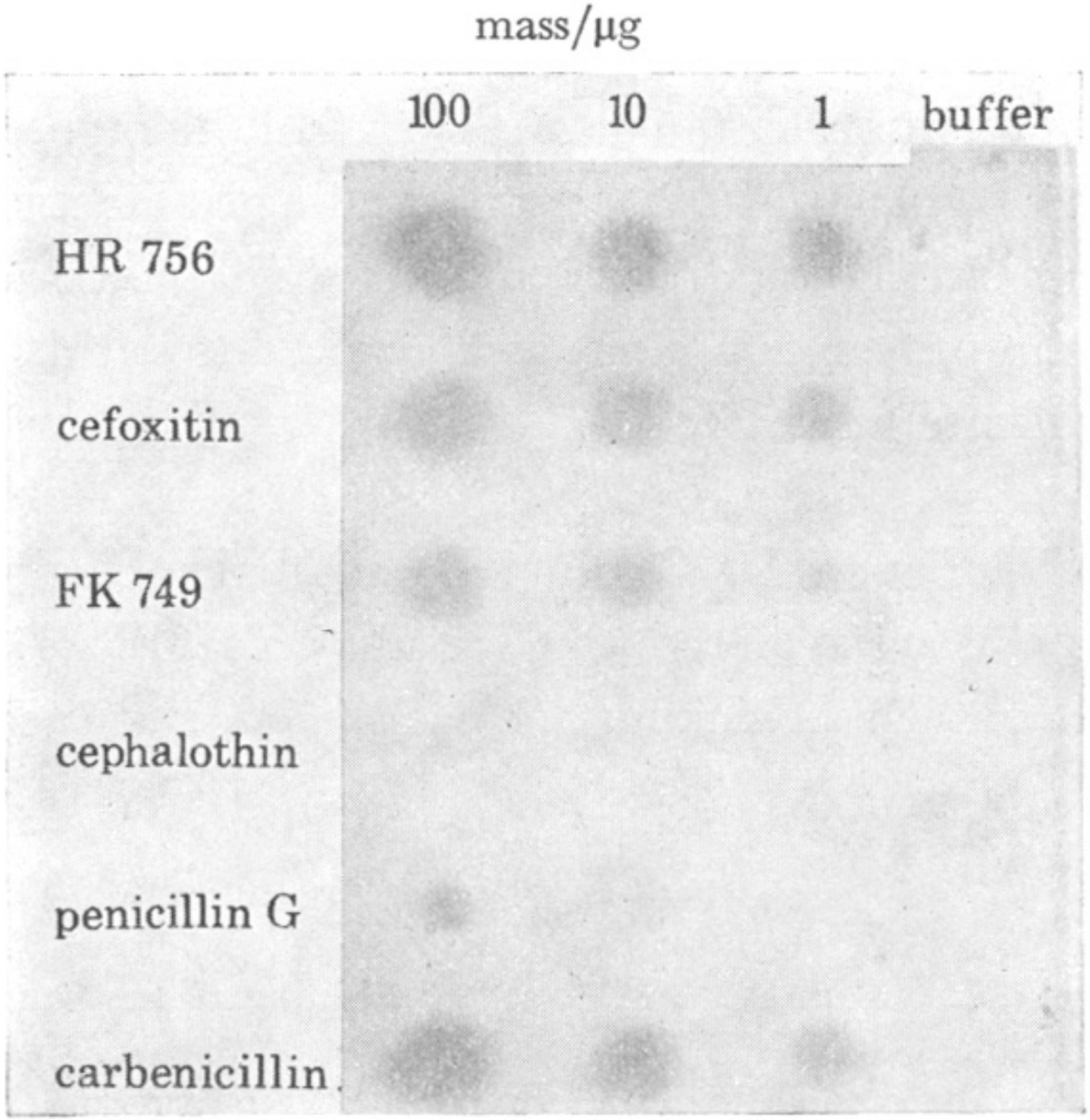

FIGURE 10. PADAC plate assay for essentially non-inactivating β-lactam compounds. Paper disks (8 mm in diameter) soaked with the indicated amount of β-lactam antibiotics were applied on top of a β-lactamase layer. After 30 min the plate was flooded with an aqueous solution of PADAC. Due to inhibition of the β-lactamase, the diffusion areas of β-lactams that are not hydrolysed during preincubation appear as violet spots on a yellow background.